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PROVISIONAL PATENT APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket Number	5853-449	Type a plus sign (+) inside this box =>	+

INVENTOR(S) / APPLICANT(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Ross	Edward	Allan	Gainesville, Florida
Batich	Christopher	David	Gainesville, Florida

TITLE OF THE INVENTION (230 characters max)

TISSUE OXYGENATION

CORRESPONDENCE ADDRESS

Stanley A. Kim, Ph.D., Esq.
Akerman Senterfitt
222 Lakeview Avenue, Suite 400
West Palm Beach, FL 33401-6183

Tel: 561-653-5000

STATE	FL	ZIP CODE	33401-6183	COUNTRY	U.S.A.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No

[ ] Yes, the name of the U.S. Government agency and the Government contract number is:

Applicant claims Small Entity Status.

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Respectfully submitted,

SIGNATURE _____ Date March 26, 2004

TYPED or PRINTED NAME Amy A. Ostrom, Ph.D. REGISTRATION NO. (if appropriate) 52,088

[ ] Additional inventors are being named on separately numbered sheets attached hereto

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TISSUE OXYGENATION

FIELD OF THE INVENTION

This invention relates to the fields of medicine, radiology, and diagnostic imaging. More particularly, the invention relates to a method for assessing oxygenation in a tissue.

BACKGROUND OF THE INVENTION

Hypoxia due to inadequate blood supply, low oxygen levels in the blood, and/or high tissue demand is a significant cause of morbidity and mortality throughout the world. Accordingly, there is a great need in clinical medicine for methods of detecting low oxygen levels in tissues. Current methods of detecting hypoxia *in vivo* include angiography and experimental magnetic resonance imaging (MRI)-based methods. Angiography utilizes x-ray technology and a contrast solution to visualize blood flow through the arteries. Although effective, angiography is also invasive and can cause undesired side-effects from use of contrast agents.

Experimental MRI-based for measuring tissue oxygenation are advantageous over angiography because they are more direct (measuring oxygen levels directly rather than by inference from blood flood) and less invasive. Moreover experimental MRI-based methods offer very good resolution. For example, "BOLD" MRI can be used to image hemoglobin oxygenation within intact red blood cells. To obtain good resolution, however, current experimental MRI methods of detecting tissue oxygen levels require the use of specialized equipment (e.g., that generate very high magnetic fields) not often found in most clinical diagnostic facilities. An MRI-based method for directly determining tissue oxygenation using standard clinical MRI equipment would therefore be of great benefit.

SUMMARY OF THE INVENTION

The invention relates to the discovery that a blood substitute bound to a water-soluble polymer can serve as an MRI contrast agent in MRI-based methods for assessing tissue oxygenation in a subject using MRI equipment (e.g., standard clinical MRI equipment). The contrast agent allows resolution of tissue oxygen levels without requiring the use of very high magnetic fields produced in experimental MRI methods. The contrast agent includes a blood substitute conjugated to a molecule that provides protons or permits interaction with protons in

an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., a water-soluble polymer).

In the experiments described below, polyethylene glycol (PEG) derivatives of bovine hemoglobin (pegylated Hb or PEG-Hb) were used as the contrast agent. The hemoglobin portion of this agent provides MR contrast because at the deoxygenated state the constituent iron is paramagnetic and provides lower relaxation times. Pegylation of Hb alters the hydration of the Hb molecule and thereby changes the proton magnetic resonance (MR) signals produced upon oxygenation/deoxygenation during MRI. In the examples described below, the nuclear MR characteristics of PEG-Hb in oxygenated and deoxygenated states were examined. Substantial changes in the T_1 and T_2 values depending on the state of oxygenation were observed. For example, using the index of the reciprocal change in T_2, there was an approximately 34% change in that value, a percentage change indicative of a successful imaging agent.

PEG-Hb is advantageous for use as a contrast agent for a number of reasons. First, it has been evaluated thoroughly for use as a blood substitute and has proven to be stable and non-toxic. Second, it is highly accessible to water protons compared to Hb contained in erythrocytes. Moreover, the polyethylene glycol portion of the molecule facilitates the diffusion of the water molecules. Third, because the PEG-Hb molecule is relatively small compared to an erythrocyte, it can pass through the walls of the capillaries into the interstitial space allowing the tissue itself to be analyzed. Thus the rate at which PEG-Hb is deoxygenated as it moves into the interstitial space can be detected using MRI. In this manner, hypoxic (ischemic) and normoxic tissue can be distinguished.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the present specification, including definitions, will control. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The particular embodiments discussed below are illustrative only and not intended to be limiting.

DETAILED DESCRIPTION OF THE DRAWINGS

FIG. 1 is a pair of graphs showing the differences in T_1 and T_2 relaxation times for the oxygenated (Oxy-Hb) and deoxygenated (Deoxy-Hb) forms of PEG-Hb as a function of time for two different samples.

DETAILED DESCRIPTION OF THE INVENTION

A preferred method of the invention for assessing oxygenation in a tissue (e.g., in a subject) utilizes PEG-HB as an MRI contrast agent. The invention is particularly advantageous for analyzing tissue oxygenation to, for example, diagnose ischemia and/or infarction. The invention can be also be used to analyze areas of dangerously low oxygenation in tissue such as the brain (e.g., after strokes or at risk of extension), heart, (e.g., after a heart attack or at risk of infarction), bowel (e.g., mesenteric ischemia), or limbs (e.g., for claudication) and to detect the presence of tumors (which are hypoxic). In addition to diagnosing, the imaging methods can be used to direct decision making for revascularization procedures. They can also be used to examine cerebral or myocardial perfusion during states of variable activity or perfusion.

The below described preferred embodiments illustrate adaptations of the invention. Nonetheless, from the description of these embodiments, other aspects of the invention can be made and/or practiced based on the description provided below.

Contrast Agent

The invention provides a contrast agent including a blood substitute (i.e., an oxygen-carrying molecule suitable for administration to an animal subject) conjugated to a molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., a water-soluble polymer). A preferred contrast agent is one whose MR imaging characteristics differ between oxygenated and deoxygenated states. Any suitable blood substitute and any suitable molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., water-soluble polymer) may be used. As examples, suitable blood substitutes include Hb, modified Hb (e.g., cross-linked, polymerized, pegylated), synthetic non-Hb-based agents, fluorocarbon compounds, and perfluorocarbon moieties. Suitable blood substitutes (e.g., Hb) can be obtained commercially. Naturally occurring blood substitutes may be isolated from animal hosts, e.g., mice, rats, rabbits, goats, sheep, pigs, horses, cattle, dogs, cats, and primates such as monkeys, apes, and human beings. A number of different blood

substitutes are described in D.R. Spahn, Critical Care 3:R91-R92, 1999. A non-exhaustive list of suitable water-soluble polymers includes PEG, dextran, albumin as well as combinations thereof.

In the examples described below, pegylated bovine Hb was used as the contrast agent. Hb is preferred as a blood substitute because the relaxation times of oxygenated and deoxygenated Hb differ, resulting in an oxygen-sensitive contrast agent. Bovine Hb is a beneficial source because it is inexpensive, readily available, may be easily modified and has increased oxygen carrying capacity. PEG, a water-soluble polymer, is preferred for use in the invention as a molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation because it imparts an improved interaction between Hb and water molecules in the tissue. PEG is a gel-like substance that facilitates the diffusion of the water molecules. The PEG polymer stabilizes the Hb protein and the resulting conjugate is non-toxic, highly accessible to water protons, and able to penetrate through interstitial space. As PEG-Hb is relatively small compared to the erythrocyte, PEG-Hb molecules are capable of passing through the walls of capillaries, thereby increasing the number of locations in which the PEG-Hb may be used as a contrast agent. In addition, the PEG polymer minimizes antigenicity and does not change the blood chemistry or the function of the cardiovascular system.

PEG-Hb may be obtained from any commercial source. Otherwise, the Hb may be purified directly from a source and then attached to a PEG-based polymer. Alternatively, the Hb may be obtained from a commercial source and then attached to a PEG-based polymer by methods known in the art. One such method is set forth in U.S. Patent No. 5,650,388. PEG-Hb is also commercially available. If obtained from a commercial source, PEG-Hb should be checked for the presence of contaminants such methemoglobin. If present, the PEG-Hb should be purified before use to ensure proper results. For example, contaminants such as methemoglobin could confound the results of MRI tests.

In alternative embodiments, the PEG-Hb of the present invention may include derivatives in which the PEG moiety or the Hb protein is modified. The PEG-Hb may be a Hb protein conjugated to PEG that has been modified with another moiety, for instance, s-nitrosylated PEG (SNO-PEG). Other useful PEG derivatives include, but are not limited to, nucleophilic PEGs, carboxyl PEGs, electrophilically activated PEGs, sulfhydryi-selective PEGs, heterofunctional PEGs, biotin PEGs, vinyl derivatives, PEG silanes and PEG phospholipids

Alternatively, the PEG-Hb may be a Hb protein conjugated to PEG in which the Hb has been modified with another moiety, for instance, pyridoxal phosphate, resulting in a pyridoxylated PEG-Hb derivative. In yet another embodiment, the PEG-Hb protein may be conjugated to an alpha-carboxymethyl, omega-carboxymethoxyl polyoxyethylene (POE), resulting in a POE conjugated PEG-Hb derivative. In each of these embodiments, the PEG-Hb is safe and non-toxic. In one embodiment, the PEG-Hb derivative of the invention is a PEG polymer conjugated to a Hb protein.

Although the invention is focused on the combination of Hb and PEG, any suitable combination of blood substitute and any molecule that provides protons or permits interaction with protons in an aqueous solution so as to alter the MR signal with oxygenation or deoxygenation (e.g., water-soluble polymer) may be used as a contrast agent in methods of the invention.

Determining An Oxygen Concentration In A Tissue

The invention also provides a method for assessing oxygenation in a tissue. The method includes the steps of providing a tissue, introducing a contrast agent to the tissue, wherein the contrast agent comprises a blood substitute conjugated to a water-soluble polymer, and subjecting the tissue to MRI. Following irradiation of the tissues, electromagnetic radiation is released from hydrogen nuclei in water molecules (i.e., relaxation). In select embodiments, the irradiation is irradiation in the radiofrequency range. The spin-lattice (T_1) and the spin-spin relaxation (T_2) times of the protons are determined. In one variation of the method, the values for T_1 and T_2 are compared for both the oxygenated and deoxygenated states of PEG-Hb to evaluate the oxygenation in tissues. In another variation of this method, the T_1 and T_2 values are utilized by MR software to reconstruct a visual image of oxygenation in a tissue. The method is expected to be useful in assessing tissue oxygenation qualitatively, as well as quantitating oxygenation levels in tissues. The method can be used in many applications, including, for example, the detection of ischemia in tissues. Other MRI techniques, such as "bold" or "fast" MRI, may be used with the contrast agents of the present invention. MRI methods are described in detail in Kuperman, V., Magnetic Resonance Imaging Physics and Biomedical Applications, Academic Press, 2000. The use of NMR imaging to examine tissue oxygenation is described in R. Vink, Adv. Exp. Med. Biol. 316:187-193, 1992 and Ogawa et al., Proc. Natl. Acad. Sci. 87:9868-9872, 1990.

Subjects

Because subjects from many different species are susceptible to low tissue oxygenation (e.g., ischemia, infarction), the invention is believed to be compatible with many types of animal subjects. Thus one example of the foregoing method includes the step of administering the contrast agent to a subject and then subjecting the subject to MRI. A non-exhaustive exemplary list of animal subjects that might be analyzed includes mammals such as mice, rats, rabbits, goats, sheep, pigs, horses, cattle, dogs, cats, and primates such as monkeys, apes, and human beings. Those animal subjects known to suffer from or suspected to suffer from low tissue oxygenation are preferred for use in the invention. In particular, human patients suffering from or suspected of suffering from low tissue oxygenation are suitable subjects for use in the invention.

Effective Amounts

The contrast agents described above are preferably administered to a mammal (e.g., human) in an effective amount, that is, an amount capable of producing a desirable result in a treated mammal (e.g., being detectable in *in vivo* imaging). Such a therapeutically effective amount can be determined as described below.

Toxicity and therapeutic efficacy of the compositions utilized in methods of the invention can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Those compositions that exhibit large therapeutic indices are preferred. While those that exhibit toxic side effects may be used, care should be taken to design a delivery system that minimizes the potential damage of such side effects. The dosage of preferred compositions lies preferably within a range that includes an ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

As is well known in the medical and veterinary arts, dosage for any one animal depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, time and route of administration, general health, and other drugs being administered concurrently. It is expected that an appropriate dosage for intravenous

administration of PEG-Hb would be in the range of about 3-5% of total blood volume (e.g., approximately 200 ml).

The compositions described above may be administered to animals including human beings in any suitable formulation by any suitable method. For example, a conventional syringe and needle can be used to inject a contrast agent formulation into a subject. Depending on the desired route of administration, injection can be *in situ* (i.e., to a particular tissue or location on a tissue), intramuscular, intravenous, intraperitoneal, or by another parenteral route. Parenteral administration of a contrast agent can be performed, for example, by bolus injection or continuous infusion.

To facilitate delivery of the contrast agent, the contrast agent may be mixed with pharmaceutically acceptable carriers or diluents such as physiological saline or a buffered salt solution. Suitable carriers and diluents can be selected on the basis of mode and route of administration and standard pharmaceutical practice. A description of exemplary pharmaceutically acceptable carriers and diluents, as well as pharmaceutical formulations, can be found in Remington's Pharmaceutical Sciences, a standard text in this field, and in USP/NF. Other substances may be added to the compositions to stabilize and/or preserve the compositions.

EXAMPLES

Example 1 - Determination of T_1 and T_2 in Oxygenated/Deoxygenated PEG-Hb Samples

Two hundred microliters of samples containing 3% PEG-Hb were prepared. For deoxygenation, 5 mg of dithionite which had been stored in a desiccator under nitrogen gas was added. The sample was then vortexed and nitrogen gas was injected from the top of the tube. To determine T_1 for the samples, an inversion recovery experiment was performed and a CPMG spin echo experiment was conducted to determine T_2. NMR Conditions: 23°C, 7 Tesla, -80 dB attenuation, spatial width: 400, line broadening: 1 Hz, 1000 points, no zero filling.

Figure 1 shows the results of the NMR experiments. After deoxygenation, T_1 values in the first sample (A) increased from an average of 2.29 sec to 2.37. The second sample (B) increased from an average of 2.06 sec to 2.13 sec, resulting in only a 3% increase in the reciprocal values of T_1 for both samples. The differences in the reciprocal values of T_2 are greater than 30%. In the first sample (A), T_2 decreased from an average of 128 msec to 95 msec,

which provided a 34% difference in the reciprocal values of T_2. The second sample (B) decreased from an average of 97 msec to 73 msec reducing the reciprocal values of T_2 to 33%.

Example 2- Target Tissues

Pegylated hemoglobin (or other blood substitute-based oxygenation imaging agents) is expected to be of broad clinical and experimental use. Such agents can be utilized to ascertain areas of dangerously low oxygenation, such as in the brain (e.g, after strokes or at risk of extension), heart (e.g, after heart attacks or at risk of infarction), bowel (e.g., mesenteric ischemia), detect the presence of tumors (which are hypoxic) or limbs (e.g., for claudication). The results of the imaging could be used diagnostically as well as be a tool to direct decision making for revascularization procedures. In experimental research it similarly could be used to study cerebral or myocardial perfusion during states of variable activity or perfusion.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspect, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for assessing oxygenation in a tissue, the method comprising the steps of:

(A) providing a tissue;

(B) introducing to the tissue a contrast agent comprising pegylated hemoglobin;

(C) placing the tissue in a magnetic field and irradiating the tissue with radio frequency energy; and

(D) determining spin-lattice and spin-spin relaxation times of water protons associated with oxygenated and deoxygenated states of the hemoglobin in the tissues.

2. The method of claim 1, further comprising the step (E) of comparing the spin-lattice or the spin-spin relaxation times of the oxygenated and the deoxygenated states of the hemoglobin to assess tissue oxygenation.

3. The method of claim 1, further comprising the step (E) of extrapolating the spin-lattice and spin-spin relaxation times of water protons to generate an image correlated with tissue oxygenation levels.

4. The method of claim 1, wherein the tissue is located in an animal subject.

5. The method of claim 4, wherein the animal subject is a human being.

ABSTRACT

An effective contrast agent for use in MRI-based methods for directly determining tissue oxygenation in a subject using MRI equipment as well as methods for assessing tissue oxygenation were discovered. Methods employ a blood substitute bound to a water soluble polymer as an MRI contrast agent. In the experiments described herein, polyethylene glycol (PEG) derivatives of bovine hemoglobin (pegylated Hb or PEG-Hb) were used as the contrast agent.

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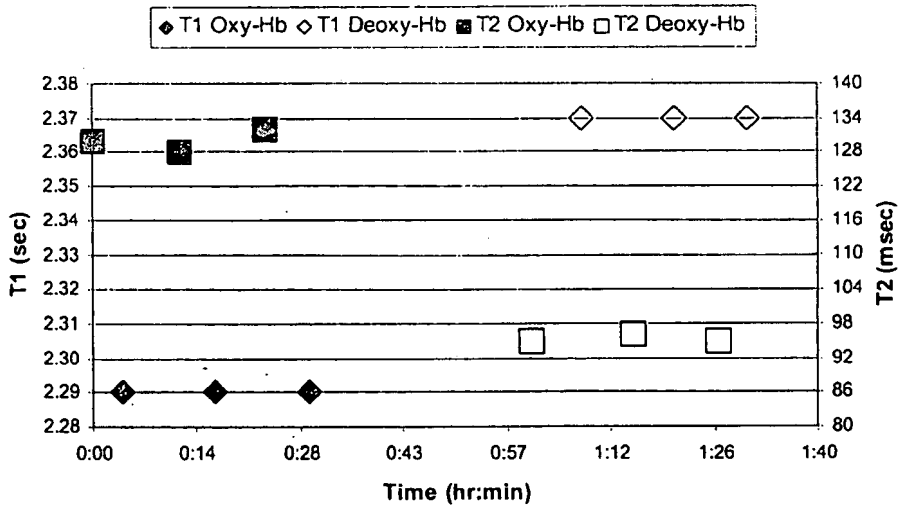
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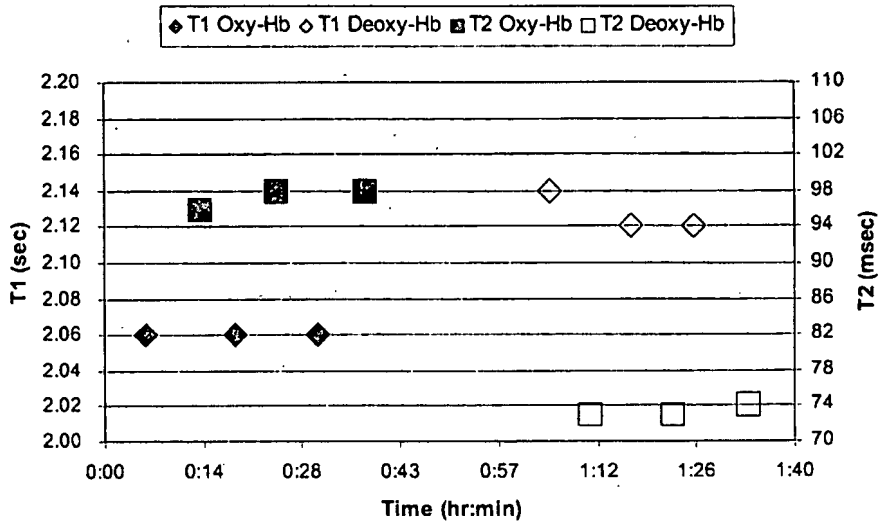
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Fig. 1